

Full Length Research Paper

Transfer of amoxicillin resistance gene among bacterial isolates from sputum of pneumonia patients attending the University of Benin Teaching Hospital, Benin city, Nigeria

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A total of 160 sputum samples were obtained from pneumonia patients (1 – 30 years) attending the University of Benin Teaching Hospital, Benin City. The microorganisms encountered included *Streptococcus viridians*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Staphylococcus* species. *S. viridians* was highest in occurrence (51.5%) and *Staphylococcus* spp. was the least (2.9%). Their antibiotic resistance pattern revealed that streptomycin had the highest activity (94.1%) and gentamycin the least (23.8%). Amoxicillin resistance gene (*amx*^r) was detected in 89 (88.1%) out of the total isolates. When these resistant isolates were subjected to curing using 10% sodium dodecyl sulfate, 73 (82.0%) lost their resistance genes. An average transfer rate of 53.88% and 33.59% were obtained for intraspecies and intergeneric transfer of *amx*^r gene respectively. Intraspecies gene transfer rate was significantly higher than intergeneric at $p < 0.001$, using the chi – square goodness of fit test for statistical analysis.

Keywords: Amoxicillin resistance, pneumonia, gene transfer, plasmid, curing, conjugation, sputum.

INTRODUCTION

Antibiotics were initially developed for the treatment of infectious diseases in people. Their powerful killing and growth inhibitory effects have reduced the number of susceptible strains (Donald, 1999; Smith et al., 2003). Individuals who are debilitated or have other predisposing factors are at higher risk of infection than healthy persons (Sheikh et al., 2003; Lisa and Rodgers, 1999). The overall volume of antibiotics prescribing is the primary factor driving resistance at both local and regional levels although other influences notably clonal spread complicate epidemiology (Goossens, 2005; Ball et al., 2002; Charlebois et al., 2002). The use of antibiotics in animal feed may have a significant effect on the development of antibiotic resistance (Barza et al., 2002). Initially, infections caused by antimicrobial resistant

bacteria occurred mainly in hospital settings where antibiotics use was most extensive. Thus bacteria carrying antimicrobial resistance genes had survival advantage that facilitated dissemination in this settings (Dzidic and Bedekovic, 2003).

Antibiotic resistance genes are often associated with conjugative plasmid or transposons which encode the proteins necessary to initiate and complete their transfer to new hosts (Nahid et al., 2007). The most common genetic instrument for resistance among bacteria is the resistance R-plasmid. (Akortha and Egbule, 2008; Okeke et al., 1999). Acquisition of these plasmid occur via all the three types of recombination (transduction, transformation and conjugation), although conjugation appears to be the most common and convenient method (Sheikh et al., 2003). The acquisition of resistance by conjugation occurs through plasmids, transposons, integrons and gene cassette (Wang et al., 2004; Yukuta et al., 2004; Yah et al., 2008). Several workers have shown that resistance genes could be transferred from

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one bacterium to the other *in vitro* and *in vivo*. Nahid *et al.* (2007) reported *in vitro* intraspecies transfer of ampicillin resistance gene between *E. coli* strains in the bowel microbiota of an infant treated with antibiotics. Akortha and Filgona (2009) demonstrated the transfer of gentamycin resistance genes among enterobacteriaceae isolated from the urinary tract of infected patients. Similarly, Aluyi and Akortha (2002) reported *in vitro* intra and inter - species transfer of ampicillin resistance genes among enteric bacteria of diarrhoea origin.

In the context of considerations for improving therapeutic outcomes, reducing resistance emergence and minimizing cost by limiting and optimizing therapy, respiratory infections are clearly an appropriate area for action (Ball *et al.*, 2002). It is important that antibiotic resistance trend be put under check through intensive research and antibiotic surveillance. With the propensity of organisms in sputum acquiring resistance determinants to various antibiotics, their ability to transfer plasmids to other species and genera of bacteria are of great concern.

This study was carried out to determine the frequency of amoxicillin resistance gene (*amx^r*) among bacterial pathogens from sputum of pneumonia patients and to demonstrate their intraspecies and intergeneric transferability.

MATERIALS AND METHODS

Sample collection

One hundred and sixty sputum samples were collected in sterile, screw capped containers. The expectorated sputum was taken by asking the patients to cough deeply into the container, followed by immediate screwing on the cap. Samples were transported to the laboratory within 2 hours and processed immediately for the prevalence of pathogens involved in pneumonia cases.

Microbiological procedures

Isolation and identification

The methods of Pye *et al.* (1995) and Cheesbrough (2002) were used for isolation. Sputum samples were homogenized with 100µg/ml of dithiothreitol and incubated at 37°C for 15 minutes to aid homogenization. 1ml of sample was diluted in sterile saline (0.9% w/v sodium chloride) to give 10⁻¹, 10⁻², 10⁻³ 10⁻⁴ and 10⁻⁵. Three dilutions (10⁻³, 10⁻⁴ and 10⁻⁵) were inoculated onto blood, chocolate and MacConkey agars and incubated at 37°C in an atmosphere of 7% CO₂ and examined for growth after 24 - 48 hours. Optochin disc was placed onto the streak of these isolates to differentiate the species of *Streptococci*. Coagulase test was performed

according to the method of Cheesbrough (2002) and used to identify *Staphylococcus aureus*.

Antibiotics susceptibility test

This was performed using the standard disk diffusion test as described by Kirby and Bauer (1966) and NCCLS (2004). The antibiotic disk (optodisc) used contained the following antibiotics: chloramphenicol (30µg), augmentin (30µg), amoxicillin (30µg), sparfloxacin (10µg), perfloxacin (30µg), ciprofloxacin (10µg), gentamicin (30µg), streptomycin (30µg), ofloxacin (30µg) and cotrimoxazole (30µg). The zones of inhibition were measured and interpreted according to the criteria of NCCLS (2004).

Plasmid curing

The method of Yah *et al.* (2008) was used. Sub-inhibitory concentration of sodium dodecyl sulfate (SDS) was used for plasmid curing. Antibiotic resistant isolates were grown at 37°C for 24 hours in nutrient broth containing 10% SDS. After which, the broth was agitated to homogenize the content and a loopful subcultured onto Mueller Hinton agar (MHA) plates. The plates were incubated at 37°C for 24 hours after which colonies were screened for antibiotic resistance by the disk diffusion method. Cured markers were determined by comparison between the pre- and post- curing antibiograms of isolates. Loss of resistance markers gave an indication that those markers were probably located on a plasmid and not on the chromosome.

Amoxicillin resistance gene transfer by conjugation

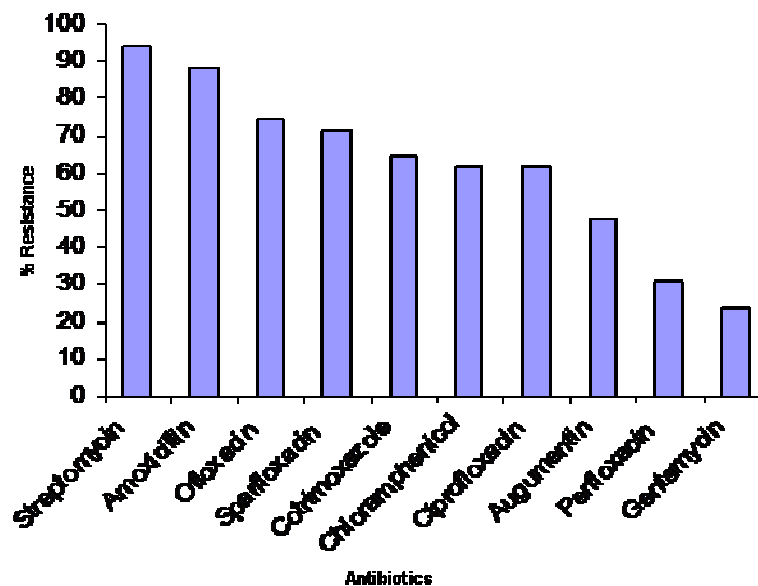
It was performed by the method of Nahid *et al.* (2007) using Luria-bertani broth. Donor isolates which were resistant to amoxicillin and sensitive to perfloxacin were used. Donor and recipient cells were grown separately in 5mls of nutrient broth at 37°C for 24 hours. Mueller Hilton agar plates containing amoxicillin (30µg/ml) and perfloxacin (30µg/ml) were prepared separately. These were used as selective markers for the transconjugants. The transconjugants were then streaked on Luria-bertani broth and incubated at 37°C for 24 hours. Their identities were confirmed by their antibiotics resistance patterns.

Statistical analysis

The chi – square goodness of fit test adopted from Ogbeibu (2005) was used to test for significant differences in the values obtained. All statistical tests were carried out using the SPSS 16.0 Windows based program.

Table 1: Incidence of bacterial isolates from sputum (%)

Bacterial isolates	No. of isolates (%)
<i>S. viridians</i>	52 (51.5)
<i>S. aureus</i>	22 (21.8)
<i>K. pneumonia</i>	14 (13.9)
<i>M. catarrhalis</i>	10 (9.9)
<i>Staphylococcus</i> spp.	3 (2.9)
Total	101 (100.0)

**Figure 1:** Percentage resistance of isolates to antibiotics**Table 2:** Prevalence of amoxicillin resistant isolates (%)

Bacterial isolates	Total no. of isolates	No. resistant (%)
<i>S. viridans</i>	52	50(56.2)
<i>S. aureus</i>	22	19(21.3)
<i>K. pneumonia</i>	14	11 (12.4)
<i>M. catarrhalis</i>	10	7 (7.9)
<i>Staphylococcus</i> spp.	3	2(2.2)
Total	101	89 (100)

RESULTS

The rate of occurrence of *Streptococcus viridans* (51.5%) was significantly higher than any other bacterial species at $p < 0.001$. This was followed by *Staphylococcus aureus* (21.8%), *Klebsiella pneumoniae* (13.9%), *Moraxella catarrhalis* (9.9%) and *Staphylococcus* spp. (2.9%) (Table 1). The isolates resistance rate towards streptomycin antibiotic (94.1%) was significantly high ($p <$

0.001). The least rate of resistance (23.8%) was recorded against gentamycin. Resistance of isolates against the quinolones (ciprofloxacin, perfloracin, ofloxacin and sparfloxacin) was also observed to be significantly high at $p < 0.001$.

Eighty –nine (88.1%) of the total isolates displayed significantly high resistance rate against amoxicillin antibiotic (Figure 2, Table 2). The rate of resistance was highest for *Streptococcus viridans* (56.2%) and lowest for

Table 3: Plasmid curing of resistant isolates

Bacterial isolates	No. subjected to curing	No. cured of only <i>amx^r</i> gene	No. cured of all resistance markers	Total no. cured (%)
<i>S. aureus</i>	19	15	1	16 (22.0)
Staphylococcus spp.	2	1	---	1 (1.4)
<i>K. pneumoniae</i>	11	8	2	10 (13.7)
<i>S. viridians</i>	50	35	5	40 (54.8)
<i>M. catarrhalis</i>	7	4	2	6 (8.2)
Total	89	63	10	73 (100)

Table 4: Intraspecies transfer of *amx^r* genes

Donor isolates (<i>amx^rpef^s</i>)	Recipient isolate (<i>amx^spef^r</i>)	No. of Transconjugant detected (%)
<i>K. pneumoniae</i> (11)	<i>K. pneumoniae</i> 33	7 (63.6)
<i>K. pneumoniae</i> (11)	<i>K. pneumoniae</i> 85	8 (72.7)
<i>K. pneumoniae</i> (11)	<i>K. pneumoniae</i> 93	6 (54.6)
<i>M. catarrhalis</i> (7)	<i>M. catarrhalis</i> 28	4 (57.1)
<i>M. catarrhalis</i> (7)	<i>M. catarrhalis</i> 52	3 (42.9)
<i>M. catarrhalis</i> (7)	<i>M. catarrhalis</i> 68	4 (57.1)
<i>S. aureus</i> (19)	<i>S. aureus</i> 5	9 (47.4)
<i>S. aureus</i> (19)	<i>S. aureus</i> 22	7 (36.8)
<i>S. aureus</i> (19)	<i>S. aureus</i> 95	10 (52.6)

Staphylococcus spp (2.2%). When the 89 amoxicillin resistant isolates were subjected to curing in the presence of 10% SDS (Table 3), seventy - three (82%) were cured of their amoxicillin resistance gene (*amx^r*). This curing rate was quite significant at $p < 0.001$ and gave an indication that the *amx^r* gene was located on a plasmid and not on the chromosome. Loss of resistance gene was total in 10 (11.2%) out of the total isolates and variable in 63 (70.8%).

Intraspecies and intergeneric transfer of *amx^r* gene were carried out on three bacterial isolates; *K. pneumoniae*, *S. aureus* and *M. catarrhalis*. Results obtained for intraspecies transfer are shown in table 4. Isolates such as *K. pneumoniae* 37, *M. catarrhalis* 61 and *S. aureus* 32, which were negative to curing transferred their *amx^r* gene to recipient species. This showed that transfer of *amx^r* gene in these strains may have been chromosomally mediated. An average intraspecies transfer rate of 53.9% was observed.

Table 5 below shows the rate of transfer of *amx^r* gene from one genus to the other. Again same isolates (which were negative to curing) transferred their *amx^r* gene to other genera. Six *K. pneumoniae* isolates (which were positive to curing) could not transfer their *amx^r* gene to other genera. This genetic marker may have been borne on non – conjugative R – plasmids in these strains. An average intergeneric transfer rate of 33.6% was

observed. Intraspecies transfer rate (53.9%) was significantly higher than intergeneric at $p < 0.001$.

DISCUSSION

The bacterial species isolated in this study included *Streptococcus viridans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Staphylococcus* spp. These were similar to those reported by earlier workers (Pye et al., 1995; Nabeetha et al., 2005; Verenkar et al., 1993; Anthony et al., 2008; Enabulele et al., 2008). Pye et al (1995) reported the presence of *Pseudomonas aeruginosa*, *Branhamella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus* in their sputum samples. Verenkar et al (1993) and Nabeetha et al. (2005) reported the presence of same organisms including *Streptococcus pneumoniae*, some Gram-positive cocci and Gram-negative enteric rods. Variations in the types of organisms isolated could be attributable to factors such as media, culturing method, time and method of sampling. Period of transportation and storage before culturing can also be causative factors.

The resistance rate was high for the quinolones (perfloracin, ciprofloxacin, ofloxacin, sparfloxacin) as observed in this study. This is in accordance with the

Table 5: Intergeneric transfer of *amx^r* genes

Donor isolates (<i>amx^r pef^s</i>)	Recipient Isolate (<i>amx^r pef^r</i>)	No. of Transconjugant detected (%)
<i>K. pneumoniae</i> (11)	<i>M. catarrhalis</i> 28	5 (45.5)
<i>K. pneumoniae</i> (11)	<i>M. catarrhalis</i> 52	4 (36.4)
<i>K. pneumoniae</i> (11)	<i>M. catarrhalis</i> 68	4 (36.4)
<i>K. pneumoniae</i> (11)	<i>S. aureus</i> 5	4 (36.4)
<i>K. pneumoniae</i> (11)	<i>S. aureus</i> 22	4 (36.4)
<i>K. pneumoniae</i> (11)	<i>S. aureus</i> 95	3 (27.3)
<i>M. catarrhalis</i> (11)	<i>K. pneumoniae</i> 33	3 (42.9)
<i>M. catarrhalis</i> (7)	<i>K. pneumoniae</i> 85	3 (42.9)
<i>M. catarrhalis</i> (7)	<i>K. pneumoniae</i> 93	2 (28.6)
<i>M. catarrhalis</i> (7)	<i>S. aureus</i> 5	3 (42.9)
<i>M. catarrhalis</i> (7)	<i>S. aureus</i> 22	3 (42.9)
<i>M. catarrhalis</i> (7)	<i>S. aureus</i> 95	2 (28.6)
<i>S. aureus</i> (19)	<i>K. pneumoniae</i> 33	7 (36.8)
<i>S. aureus</i> (19)	<i>K. pneumoniae</i> 85	6 (31.6)
<i>S. aureus</i> (19)	<i>K. pneumoniae</i> 93	4 (21.1)
<i>S. aureus</i> (19)	<i>M. catarrhalis</i> 28	4 (21.1)
<i>S. aureus</i> (19)	<i>M. catarrhalis</i> 52	5 (26.3)
<i>S. aureus</i> (19)	<i>M. catarrhalis</i> 68	4 (21.1)

findings of Enabulele et al (2006) in Benin City, South-South Nigeria but disagrees with that of Adamu et al (2009) who observed a low resistance rate of isolates against the quinolones. This goes to show that regional differences could play a role in the resistance profile of bacteria and further justifies the need to undertake regular antibiotic susceptibility studies on bacterial isolates from different geographical areas. The high resistance rate (94.1%) observed for Streptomycin in this study is in agreement with the high resistance rate (79%) reported by Iwaiokun et al (2001) but disagrees with the relative low resistance rate (54%) reported by Sheikh et al., 2003. The high level of resistance against Streptomycin antibiotic is probably due to abuse of the drug since it is frequently recommended for the treatment of respiratory tract infections and is readily available across the counter in tablet form. This has led to decrease in its effectiveness with subsequent increase in resistance rate.

The observed rate of resistance of isolates to gentamycin was quite low (23%). This agrees with the reports of Yah et al. (2007) and Akortha and filgona (2009) who reported low resistance rates of 17.9% and 17.7% respectively for same antibiotic. This low resistance rates reported for gentamycin may be due to the fact that this drug is available in ampules and can only be administered intravenously making intake not as palatable as oral antibiotics. The results of this study confirms *in vitro* bacteriological efficacy of gentamycin as reported by earlier workers (Olukoya and Oni, 1990 and

Iwaiokun et al., 2001). Gentamycin therefore remains the drug of choice for the treatment of pneumonia as most pneumococcal agents are susceptible to it. Generally high resistance rates were observed for nearly all the antibiotics tested. This has serious implications for the therapy of respiratory tract infections. There is a general abuse of commonly used antibiotics by patients through the purchase of expired drugs, purchase of incomplete doses and taking of antibiotics without prescription.

The high amoxicillin resistance rate (88.1%) observed in this study is comparable to that of other workers. A resistance rate of 92.2% and 70% were reported by Akortha and Egbule (2008), Diano and Akano (2009) respectively. There is serious need for control of antibiotic resistance which if left uncontrolled might lead to serious health hazards. The indiscriminate use of antibiotics and the discontinuation of antibiotic use when signs and symptoms disappear before the pathogen is finally eliminated are some factors responsible for high rate of resistance. The different resistance pattern observed suggests the dynamic adaptation by bacteria in response to antibiotic treatment which occur readily. The multiple antibiotic resistances observed in this work make it difficult to ascribe any particular resistance pattern to any source as the pattern cuts across all the sources.

The curing experiment result in this study showed that the amoxicillin resistance gene (*amx^r*) gene was lost in 82% of the resistant isolates when treated with 10% sodium dodecyl sulfate. This is comparable to the findings of various researchers. Gonzalez et al (1991)

reported the curing of some Enterotoxigenic *Escherichia coli* (ETEC) with the use of acridine orange while Enabulele et al (1993) reported a curing rate of 13.6% among some Gram negative bacteria using same curing agent. Similarly, Akortha and Egbule (2008) and Akortha and Filgona (2009) reported a curing rate of 79% and 76% respectively with the use of 75µg/ ml acridine orange. Other types of curing agents which had been successfully used by researchers include proflavin, phenolic compounds and ethidium bromide (Sheikh et al., 2003).

Elimination of plasmids with dyes inhibits plasmid replication and results in plasmid free segregants during subsequent cell division (Akortha and Aluyi, 2002). Curing of resistance plasmids with dyes gives an indication that the mechanism of resistance is plasmid-mediated. This resistance mechanism is of great importance in the chemotherapy of pneumonia infections since the rate of antibiotic resistance is usually high in such strains due to the ease of horizontal transferability of resistance determinants.

In this study, in vitro intraspecies and intergeneric transfer of *amx*^r gene by conjugation was observed in *K. pneumoniae*, *M. catarrhalis* and *S. aureus* suggesting that they possess conjugative plasmids. However, transfer of *amx*^r genes was not successful in some isolates that had their *amx*^r genes on plasmids. This is an indication that the *amx*^r genes in these isolates are probably located on non-conjugative plasmid. Also transfer of *amx*^r genes were observed in some isolates that had their *amx*^r genes on the chromosomes. This may be due to the fact that the *amx*^r genes were located on transposable element or integron thus resulting in transfer function. The average intra species and inter-generic transfer rates from this study were 53.9% and 33.9% respectively. Akortha and Filgona (2009) reported 41.1% and 34.8% for intra-species and inter-generic transfer respectively. Aluyi and Akortha (2002) reported transfer efficiency of 33% from some enteric bacteria of diarrhoea origin to *E. coli* (UB5201). The inter-generic transfer rate in the present study is comparatively lower than intra species transfer rate. This could be as a result of fertility inhibition, incompatibility, inability to synthesize adhesion, narrow host range (Akortha and Filgona, 2009; Tolmasky, 1990).

The presence of plasmid mediated amoxicillin resistance among organisms in sputum and it's evidence of it's transferability of *amx*^r gene between it's species and genera implies that under favourable condition, conjugal transfer of R – plasmids could occur *in vivo*. Transfer was not successful in all strains, although positive during curing procedures. The plasmids or the genetic capabilities may have been lost during repeated successive sub-cultures of the isolates. It may also be due to the possession of non-conjugative plasmids or the plasmids may have been too heavy for transfer. (Yah et al., 2008).

There is need for consistent on-going antimicrobial

surveillance for important and commonly isolated clinically significant pathogens. This could form the basis for developing and implementing measures that can reduce the burden of antimicrobial resistance and prevent a probable impending public health problem.

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